Remarks

Reconsideration of this Application is respectfully requested.   Applicants respectfully request entry of this amendment after a final Office Action because the amendments to the claims place the application in condition for allowance or better form for appeal and are filed concurrently with a Request for Continued Examination under 37 C.F.R. § 1.114. 37 C.F.R. § 1.116(a) and (b)(1).

According to the Advisory Action, the Examiner did not enter the amendments to the claims filed on October 22, 2010. Upon entry of the foregoing amendments, claims 63-77 are pending in the application, with claims 63 and 70 being the independent claims. Claims 1-62 were previously cancelled. Claims 65, 68, 70 and 71 have been withdrawn. Claims 63 and 70 have been amended to clarify the claim language as described below. New claims 72-77 are sought to be added to claim additional embodiments of independent claims 63 and 70. Support for new claims 72-77 can be found throughout the original specification, for example, at page 13, lines 21-23. As such, these changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendments and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

I.   *Rejection Under 35 U.S.C. § 112, First Paragraph - Written Description*

The Examiner maintained the rejection of claims 64, 66, 67 and 69 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description

requirement. *See* Office Action at pages 2-4. Applicants respectfully traverse the rejection for at least the reasons of record and the additional reasons that follow.

### A.	Legal Principles

The test for the written description requirement is whether one skilled in the art can reasonably conclude that the inventor has possession of the claimed invention in the specification as filed. *Ariad v. Lilly*, No. 2008-1248 (Fed. Cir., March 22, 2010); MPEP § 2163.02. The Federal Circuit has re-emphasized the well-settled principle of law that "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [they] invented what is claimed.'" *Union Oil of Cal. v. Atlantic Richfield Co.*, 208 F.3d 989 (Fed. Cir. 2000). Furthermore, an Applicant is not required to explicitly describe the subject matter. *Unocal*, 208 F.3d at 1000; MPEP § 2163.02 ("The subject matter of the claim need not be described literally (*i.e.*, using the same terms or *in haec verba* in order for the disclosure to satisfy the description requirement.").

### B.	Immunogenic Compositions of Claims 64 and 67

At page 2 of the Office Action, the Examiner states that:

> [r]egarding applicants['] comments, the cited passages of the specification are not drawn to immunogenic compositions containing a pharmaceutically acceptable carrier....The specification discloses vaccines or pharmaceutical compositions containing a pharmaceutically acceptable carrier, but does not disclose the scope of claim 64 which is a composition other than vaccine or pharmaceutical containing pharmaceutically acceptable carriers.

Applicants maintain that the immunogenic compositions of claims 64 and 67 are supported by the specification. For example, the original title of the application, "Inducing Cellular Immune Responses to Hepatitis B Virus Using Peptide and Nucleic Acid Compositions," clearly indicates that the peptide compositions of the invention are immunogenic because they are described as inducing cellular immune responses to HBV. Also, Example 3 describes various confirmatory assays performed to test the immunogenicity of the peptides of the invention. *See, e.g.*, pages 64-66. More specifically, the peptides were screened for the induction of primary antigen-specific cytotoxic T cells (CTL) *in vitro* using human peripheral blood mononuclear cells (PBMC) (*see* Tables XXXa-XXXc, "primary" CTL), and HLA transgenic mice were used to evaluate peptide immunogenicity (*see* Tables XXXa-XXXc, "transgenic" CTL). For CTL induction testing, peptides of the invention and a helper epitope hepatitis B virus (HBV) core 128 were emulsified in Freund's Incomplete Adjuvant (FIA) and injected subcutaneously into the mice. FIA is routinely used in peptide epitope compositions administered to humans, and therefore one of ordinary skill in the art would consider FIA "pharmaceutically acceptable." *See, e.g.*, Wang *et al., Clin. Cancer Res.*, 5:2756-2765, 1999 (copy enclosed as Exhibit 1).

Peptides of the invention were also tested for the ability to stimulate recall CTL responses in acutely infected HBV patients. *See* Tables XXXa-XXXc, "patient" CTL. For these tests, PBMC from patients acutely infected with HBV were cultured in the presence of peptides of the invention for 7 days, restimulated with peptide, and then assayed for cytotoxic activity on day 14. *See* page 65, lines 16-18 of the specification. Because these tests include culturing PBMC over 14 days, one of ordinary skill in the art

would have understood that the PBMC culturing conditions were physiologically acceptable, and therefore the peptides of the invention introduced into the culture were administered in a composition comprising a pharmaceutically acceptable carrier.

Of the peptides tested in Example 3, the vast majority were found to be immunogenic in at least one of the systems utilized. *See* Tables XXXa-XXXc ("overall") and page 65, lines 20-32 of the specification. Additionally, Tables XXXIa and XXXIb report the testing of the peptides recited in claim 63 performed by the same methods. *See also* specification at page 66, lines 7-27. Again, of these peptides tested, the vast majority were found to be immunogenic. *See* Tables XXXIa and XXX1b ("overall") and page 66, lines 10-12 of the specification. Applicants note that the mice in these experiments were not specifically evaluated for the treatment or prophylaxis of HBV. Hence, the tested compositions were, in fact, "immunogenic" compositions. Taken together, these results clearly provide that peptides of the invention, in compositions comprising a pharmaceutically acceptable carrier, are immunogenic. Therefore, this disclosure provides further support for the claimed immunogenic compositions containing a pharmaceutically acceptable carrier.

Additional support for compositions comprising an HBV epitope or peptide of the invention can be found at page 9, lines 22-28 and page 43, lines 11-15 of the specification. Support for peptides and corresponding nucleic acid compositions of the present invention is also provided at page 10, lines 16-18 of the specification. Support for compositions and combinations of compositions that enable epitope-based vaccines that are capable of interacting with multiple HLA antigens to provide broader population coverage can be found at page 10, lines 27-30 of the specification.

In addition, as noted in the Reply Under 37 C.F.R. § 1.111 filed on March 23,

2010, the specification provides that:

> [o]nce appropriately immunogenic epitopes have been
> defined, they can be sorted and delivered by various means,
> herein referred to as "vaccine" compositions.

Specification at page 41, line 33 to page 42, line 2.

An Applicant is entitled to be his own lexicographer. MPEP § 2111.01(IV). The

above passage clearly and unambiguously discloses immunogenic epitopes, such as the

immunogenic epitope peptides being claimed. Further, the above passage clearly

discloses that these claimed immunogenic epitope peptides can be delivered in various

ways, one of which disclosed is the administration with pharmaceutically acceptable

carriers. In addition, the above passage clearly discloses that the claimed immunogenic

epitope peptides which are delivered in various ways are included within the term

"vaccine" compositions. As such, in view of this definition of a "vaccine" composition

in the context of the specification, the disclosures in the specification related to a

"vaccine" composition clearly include compositions comprising the immunogenic

peptides of the invention.

For at least these reasons, Applicants submit that the specification describes the

immunogenic compositions of claims 64 and 67 such that one skilled in the art could

reasonably conclude that the inventor had possession of the claimed invention. *Vas-Cath,*

Inc. v. Mahurkar, 935 F.2d 1555, 1563 (Fed. Cir. 1991).

C.   *One or More Second Peptides of Claims 66 and 69*

At page 3 of the Office Action, the Examiner maintains that there is no support

for the recitation of "wherein said one or more second peptides is a cytotoxic T cell

(CTL)-inducing peptide or a helper T cell (HTL)-inducing peptide" because the passages cited previously by the Applicants are limited to vaccine compositions, while the claims are directed to non-vaccine compositions.

As discussed above, support for the claimed immunogenic compositions containing a pharmaceutically acceptable carrier is provided in the specification, and the disclosures in the specification related to "vaccine" compositions clearly include compositions comprising the immunogenic epitopes of the invention. In addition, the specification provides that PADRE™ universal helper T cell (HTL) epitope peptides can be included in compositions together with the peptides of the invention. *See, e.g.,* specification at page 43, lines 18-24 Applicants maintain that the specification supports the concept that multiple peptide epitopes, CTL and/or HTL, can be combined in the immunogenic compositions of the present claims for at least the reasons of record (*see, e.g.,* page 8, lines 12-14 and page 84, lines 27-32 of the specification). In view of this support, Applicants respectfully submit that the specification describes the immunogenic compositions of claims 66 and 69 such that one skilled in the art could reasonably conclude that the inventor had possession of the claimed invention.

D.   *Pan-DR-Binding Epitope of Claim 69*

At page 3 of the Office Action, the Examiner also maintains that there is no support for the immunogenic composition of claim 69 because the passages cited previously by the Applicants are limited to vaccine compositions, while the claims are directed to non-vaccine compositions.

As discussed above, support for the claimed immunogenic compositions containing a pharmaceutically acceptable carrier is provided in the specification, and the

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disclosures in the specification related to "vaccine" compositions clearly include compositions comprising the immunogenic epitopes of the invention. Applicants also maintain that the specification describes an immunogenic peptide comprising a pan-DR binding epitope. Specifically, the specification discloses:

> [i]n certain embodiments, the T helper peptide is one that is recognized by T helper cells present in the majority of the populations. This can be accomplished by selecting amino acid sequences that bind to many, most, or all of the HLA class II molecules. These are known as "loosely HLA-restricted" or "promiscuous" T helper sequences. ... For instance, a pan-DR-binding epitope peptide having the formula: aKXVWANTLKAAa, where "X" is either cyclohexylalanine, phenylalanine, or tyrosine, and a is either D-alanine or L-alanine [SEQ ID NO: 3877], has been found to bind to most HLA-DR alleles, and to stimulate the response of T helper lymphocytes from most individuals, regardless of their HLA type.

Specification at page 50, line 28 to page 51, line 12.

Thus, the specification not only describes the functional attributes of a pan-DR binding epitope, but also provides examples of well-known and frequently-utilized pan-DR binding epitopes, including the epitope specified in claim 69. In view of this support, Applicants respectfully submit that the specification describes claim 69 such that one skilled in the art could reasonably conclude that the inventor had possession of the claimed invention.

For at least the reasons above, Applicants therefore respectfully assert that the present claims satisfy the written description requirement of 35 U.S.C. § 112, first paragraph, and request that the rejection be withdrawn.

II.   Rejection Under 35 U.S.C. § 112, First Paragraph - Enablement

Claims 64 and 67 remain rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. *See* Office Action at pages 4-9. Applicants traverse the rejection for the reasons of record.

A.   Legal Principles

In order for a claim to be enabled, the specification must teach one of ordinary skill in the art to make and use the invention without undue experimentation. The factors that can be considered in determining whether an amount of experimentation is undue have been set forth in *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). Among these factors are: (1) the guidance provided by the specification; (2) the amount of pertinent literature; (3) the presence of working examples; and (4) the predictability of the art. The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine. *See id.*

B.   The disclosures in the specification support the claimed immunogenic compositions containing a pharmaceutically acceptable carrier, and relate to "vaccine" compositions that include compositions comprising the immunogenic epitopes of the invention.

At page 4 of the Office Action, the Examiner maintains that the specification is not enabling for the claimed immunogenic compositions because it does not disclose how to use the instant invention for the *in vivo* treatment/prevention of the HBV in humans and the state of the art with regard to the treatment/prevention of the HBV in humans is unpredictable. Applicants traverse the rejection for the reasons of record and for the following additional reasons. As discussed above, support for the claimed immunogenic compositions containing a pharmaceutically acceptable carrier is clearly provided in the specification, and the disclosures in the specification related to "vaccine"

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compositions clearly include compositions comprising the immunogenic epitopes of the invention. Therefore, the disclosures in the specification related to the administration of "vaccine" compositions to treat/prevent HBV (*e.g.*, page 52, line 20 to page 53, line 11) clearly include the administration of compositions to treat/prevent HBV and teach one of ordinary skill in the art how to make and use the claimed immunogenic compositions without undue experimentation.

> **C.   The Examiner's allegations that compositions containing a single HBV peptide are not known and would require undue experimentation do not support a finding of non-enablement of the present claims.**

At page 6 of the Office Action, the Examiner maintains that there is no currently known pharmaceutical composition containing a single HBV peptide for treating or preventing HBV in humans and undue experimentation would therefore be required to use the claimed compositions. Applicants respectfully disagree for at least the reasons of record.

Specifically, in view of related case law precedent, the issue of whether or not compositions containing a single HBV peptide are known in the art does not support a finding of non-enablement of the present claims. For example, in *Falkner v. Inglis*, 448 F.3d 1357 (Fed. Cir. 2006) (copy provided as Exhibit A of the Reply Under 37 C.F.R. § 1.111 filed on March 23, 2010), claims directed to a vaccine comprising a defective poxvirus were found enabled by the Court of Appeals for the Federal Circuit, even though, per the Appellant, "vaccines based on vaccinia (a type of poxvirus) had not yet been produced." *See* Exhibit A at page 6. In addition, the Federal Circuit affirmed the Board of Patent Appeals and Interference's finding that:

> ... the mere fact that the experimentation may have been difficult and time consuming does not mandate a conclusion

that such experimentation would have been considered to be "undue" in this art. Indeed, great expenditures of time and effort were ordinary in the field of vaccine preparation.

See Exhibit A at page 12.

Therefore, in view of this case law precedent, the Examiner's allegations that compositions containing a single HBV peptide were not known in the art do not support a finding of non-enablement of the present claims. Also, the experimentation required to make such compositions, despite involving great expenditures of time and effort under ordinary circumstances, is not "undue" in the relevant field and also does not support a finding of non-enablement of the present claims.

> D.   *The Examiner's allegations that one embodiment of the claimed compositions containing a single HBV peptide may not elicit a CTL response in most individuals do not support a finding of non-enablement of the present claims.*

At page 7 of the Office Action, the Examiner maintains that one embodiment of the claimed compositions containing a single HBV peptide of the present claims may not bind to most HLA alleles and therefore may not elicit a CTL response in most individuals. Applicants respectfully disagree for at least the reasons of record. As an initial matter, Applicants note that the claim is not limited to compositions containing a single HBV peptide. Also, in order to comply with 35 U.S.C. § 112, first paragraph, it is not necessary to enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment, absent a claim limitation to that effect. *See* MPEP § 2164, citing *CFMT, Inc. v. Yieldup Int'l Corp.*, 349 F.3d 1333, 1338 (Fed. Cir. 2003). Thus, the Examiner's allegations that a certain embodiment of the present claims may not elicit a CTL response in most individuals does not support a finding of non-enablement of the present claims because other embodiments of the claims will elicit a CTL

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response. *See* MPEP § 2164.08(b) ("The presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled."); *see also Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569 (Fed. Cir. 1984).

For at least the reasons above, Applicants therefore assert that the present claims satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph.

III.   Rejection Under 35 U.S.C. § 102

Claims 63, 64, 66 and 67 remain rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by U.S. Patent No. 5,360,714 ("Seeger"), as evidenced by Pasek *et al.* ("Pasek"). *See* Office Action at pages 9-10. Applicants traverse the rejection for the reasons of record and for at least the following additional reasons.

A.   Legal Principles

A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631 (Fed. Cir. 1987); MPEP § 2131. As stated by the Federal Circuit in *PPG Indus., Inc. v. Guardian Indus. Corp.*, 75 F.3d 1558, 1566 (Fed. Cir. 1996): "[t]o anticipate a claim, a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter." The absence of any claimed element from the reference negates anticipation. *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1574 (Fed. Cir. 1984). Furthermore, if an independent claim is not fully met by an alleged prior art reference, neither are the more limited dependent claims. *Application of Royka*, 490 F.2d 981, 983-984 (Cust. & Pat. App. 1974).

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B. **Seeger does not disclose all of the limitations of the present claims, and therefore does not anticipate the present claims.**

As a preliminary matter, the Examiner maintains that "[i]n view of newly submitted withdrawn claim 70, claim 63 is interpreted as encompassing the peptide recited in the claim attached to another peptide(s)." Office Action at page 8. Applicants respectfully disagree for the reasons of record and for at least the following additional reasons.

As amended, claim 63 recites peptides of a specified length that are "isolated" (*i.e.*, "An isolated peptide consisting of at most 14 amino acid residues in length, which comprises an oligopeptide selected from the group consisting of...."). Furthermore, the specification provides that the term "isolated" refers to:

> material which is substantially or essentially free from components which normally accompany the material as it is found in its native state. Thus, isolated peptides in accordance with the invention preferably do not contain materials normally associated with the peptides in their *in situ* environment.

Specification at page 12, line 32 to page 13, line 2.

Accordingly, the isolated peptides of claim 63 cannot be more than 14 amino acids in length and they must be substantially or essentially free from components which normally accompany the amino acid sequence in its native state. Therefore, the language of claim 63 is not consistent with the Examiner's interpretation that the peptides of claim 63 encompass the specific peptides recited in the claim attached to another peptide.

Further, Applicants disagree with the Examiner's reliance on claim 70 for his interpretation of claim 63. Withdrawn claim 70 is related to a composition comprising a peptide of claim 63 joined to a CTL or HTL inducing peptide. The fact that a separate

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claim directed to a genus of peptides having no more than 14 amino acids in length that are joined to something else (*e.g.*, claim 70) does not render a claim to the genus of peptides itself being longer than 14 amino acid residues (*e.g.*, claim 63). Applicants assert that such a conclusion is clearly improper, as it is essentially the equivalent of saying that the definition of a building brick reads on a house, because houses are made of bricks.

Seeger discloses a peptide sequence that *comprises* the amino acid sequence specified in the claims, along with additional amino acid sequences that flank the epitope. *See* Seeger, col. 10, 3rd paragraph; col. 5, 3rd paragraph; and cols. 11-12. Because these flanking sequences are normally associated with the epitope in its *in situ* environment, the disclosure of the epitope with the flanking sequences *cannot* be considered an "isolated" peptide of claim 63. Also, it is clear from the specification that the claimed invention concerns peptide epitopes which are fragments of antigenic proteins, and not the entire protein. *See, e.g.*, page 6, lines 21. For at least these reasons, Seeger does not disclose the exact peptide of claim 63 and therefore does not anticipate claim 63.

Claims 64, 66 and 67 depend, either directly or indirectly, from claim 63, and therefore incorporate all of the limitations of claim 63. *See* 35 U.S.C. § 112, fourth paragraph. As discussed above, Seeger does not disclose the exact peptide of claim 63. Dependent claims 64, 66 and 67 incorporate the limitations of claim 63, and therefore, Seeger does not disclose all of the limitations of any one of claims 64, 66 and 67. For at least the reasons above, Seeger also does not disclose all of the limitations of new claims 72-77 which depend from and incorporate the limitations of claim 63 or 70.

Thus, Applicants assert that Seeger does not teach all of the limitations of any one of claims 63, 64, 66 and 67 or new claims 72-77. Consequently, Seeger does not anticipate the present claims.

Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Second Amendment and Reply is respectfully requested.

Respectfully submitted,

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Exhibit 1

Phase I Trial of a MART-1 Peptide Vaccine with Incomplete Freund's Adjuvant for Resected High-Risk Melanoma[1]

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Catherine Kuniyoshi, Lucy Spears,
Georgia Jeffery, Verna Marty, Susan Groshen,
and Jeffrey Weber[2]

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ABSTRACT

Twenty-five patients with high-risk resected stages IIB, III, and IV melanoma were immunized with a vaccine consisting of the minimal epitope, immunodominant 9-amino acid peptide derived from the MART-1 tumor antigen (AAGIGILTV) complexed with incomplete Freund's adjuvant. The last three patients received the MART-1$_{27-35}$ peptide with incomplete Freund's adjuvant mixed with CRL 1005, a block copolymer adjuvant. Patients were immunized with increasing doses of the MART-1$_{27-35}$ peptide in a Phase I trial to evaluate the toxicity, tolerability, and immune responses to the vaccine. Immunizations were administered every 3 weeks for a total of four injections, preceded by leukapheresis to obtain peripheral blood mononuclear cells for immune analyses, followed by a post-vaccine leukapheresis 3 weeks after the fourth vaccination. Skin testing with peptide and standard delayed-type hypersensitivity skin test reagents was also performed before and after vaccinations. Local pain and granuloma formation were observed in the majority of patients, as were fevers or lethargy of grade 1 or 2. No vaccine-related grade III/IV toxicity was observed. The vaccine was felt to be well tolerated. Twelve of 25 patients were anergic to skin testing at the initiation of the trial, and 13 of 25 developed a positive skin test response to the MART-1$_{27-35}$ peptide. Immune responses were measured by release of IFN-γ in an ELISA assay by effector cells after multiple restimulations of peripheral blood mononuclear cells in the presence of MART-1$_{27-35}$ peptide-pulsed antigen-presenting cells. An ELISPOT assay was also developed to measure more quantitatively the change in numbers of peptide-specific effector cells after vaccination. Ten of 22 patients demonstrated an immune response to peptide-pulsed targets or tumor cells by ELISA assay after vaccination, as did 12 of 20 patients by ELISPOT. Nine of 25 patients have relapsed with a median of 16 months of follow-up, and 3 patients in this high-risk group have died. Immune response by ELISA correlated with prolonged relapse-free survival. These data suggest a significant proportion of patients with resected melanoma mount an antigen-specific immune response against a peptide vaccine and support further development of peptide vaccines for melanoma.

INTRODUCTION

The earliest hint that tumor cells were immunogenic, or expressed antigens that were recognized by the immune system, came from the work of Priehn and Main (1), who demonstrated that mice immunized with tumor cells from UV- or carcinogen-induced tumors would reject a subsequent challenge of live tumor cells from the parental tumor but not an unrelated tumor. Rejection of antigen-expressing tumor cells was mediated by specific host cytolytic T cells in UV-induced tumors (2). Tumor cells, therefore, expressed antigens that were recognized by the immune system of the tumor-bearing host. There is an accumulating body of evidence to suggest that many tumors in experimental model systems and from cancer patients express molecules that are recognized by T cells. The molecular cloning of a tumor-specific antigen expressed by a murine cell line that has been mutagenized has been described, as well as the cloning of a naturally occurring tumor-specific antigen expressed by the murine mastocytoma P815 (3, 4).

In patients bearing metastatic melanomas, a number of groups have demonstrated the existence of antitumor CTL responses. PBMCs,[3] as well as TILs, contain populations of cells and individual clones that demonstrate tumor specificity; they lyse autologous tumor cells but not natural killer targets, allogeneic tumor cells, or autologous fibroblasts (5–7). Tumor-specific TILs that mediate partial and complete regressions of metastatic melanoma after adoptive transfer with IL-2 as well as melanoma-specific CTL clones raised from the peripheral blood of melanoma patients have been used in cloning strategies to identify antigens including MAGE-1 and MAGE-3, GAGE-1, MART-1, gp100, gp75 (TRP-2), tyrosinase, mutated p16, and E-cadherin (8–20), which expands the repertoire of molecules to use in a vaccine strategy for melanoma. Eight or nine amino acid peptide epitopes have been shown to be displayed in association with class I MHC molecules for recognition by T cells (21, 22),

[3] The abbreviations used are: PBMC, peripheral blood mononuclear cell; TIL, tumor-infiltrating lymphocyte; IL, interleukin; HLA, human leukocyte antigen; NCI, National Cancer Institute; FLU, influenza; DTH, delayed-type hypersensitivity; PHA, phytohemagglutinin; IFA, incomplete Freund's adjuvant.

and tumor cells have been shown to express these naturally processed epitopes. We immunized patients with resected melanoma at high risk of harboring microscopic disease to augment T-cell immunity against a known tumor antigen. In this report, we describe the results of a Phase I clinical trial in which the minimal epitope immunodominant 9-amino acid peptide derived from a melanoma differentiation antigen, MART-1, was combined with an oleic oil-based adjuvant, Montanide ISA 51, or IFA to immunize patients with resected melanoma at high risk of harboring microscopic disease and thus at high risk of relapse. Twenty-two patients received the MART-1$_{27-35}$ peptide with IFA, and 3 had a block copolymer adjuvant added to the peptide-IFA combination. The toxicity, tolerability, and specific immune responses to the vaccine were measured, as well as baseline nonspecific immunological parameters.

MATERIALS AND METHODS

Trial Eligibility. All patients had resected stages IIB, III, and IV melanoma by the 1988 modified American Joint Commission on Cancer staging system and were rendered free of disease surgically. They were required to have a magnetic resonance image or computed tomography scan of the head, and computed tomography scans of the chest, abdomen, and pelvis showing no indication of disease within 4 weeks of initiating therapy to verify that they were clinically free of melanoma. Eligibility criteria included age 18 or greater, creatinine <1.4 mg/dl, bilirubin <1.5 mg/dl, platelets of 100,000/mm^3 or more, hemoglobin ≥9 g/dl, and total WBC of ≥3,000/mm^3. HIV, hepatitis C antibody, and hepatitis B surface antigen were required to be negative, and all patients were HLA-A2 positive by a microcytotoxicity assay. All patients were required to comprehend and sign an informed consent form approved by the National Cancer Institute and the Los Angeles County and University of Southern California Institutional Review Board.

Peptide. The MART-1$_{27-35}$ peptide (AAGIGILTV) vaccine was administered as outpatient therapy. The bulk peptide was supplied by Chiron Mimetope, Inc., and the finished injectable dosage form was manufactured by the Monoclonal Antibody/Recombinant Protein Production Facility, NCI (Frederick, MD). Peptide was provided by Cancer Therapy Evaluation Program/NCI (Bethesda, MD) under an Investigational New Drug application held by the NCI as the trifluroacetate salt in DMSO. The vials of peptide contained no preservative.

Adjuvants. Montanide ISA-51 (IFA) was manufactured by Seppic, Inc. and supplied as glass ampules containing 3 ml of sterile adjuvant solution without preservative.

CRL 1005 is a nonionic block copolymer consisting of two chemical components: hydrophobic polyoxypropylene and hydrophilic polyoxyethylene. The copolymer forms small (500 nm–2 μm) particles that combine with protein and peptide antigens. It was manufactured and supplied by Vaxcell, Inc. (Norcross, GA) as 75 mg/ml CRL 1005 in a 2.5-mg vial without preservative.

Vaccine Preparation and Administration. An appropriate amount of MART-1$_{27-35}$ was diluted with sterile DMSO (RIMSO, Gaithersburg, MD) and added in a 1:1 volume to Montanide ISA-51 and then mixed in a Vortex mixer (Fisher, Inc., Alameda, CA) for 10 min at room temperature. The resulting emulsion was injected deeply s.c. in the lateral thigh in a volume of 1 or 2 ml using a glass syringe. s.c. as opposed to intradermal administration was chosen because of the large volume of injectate (up to 2 ml). Alternating thighs were used for a total of four injections, which were done 3 weeks apart. Twenty-three patients had a leukopheresis with an exchange of ~5 liters of blood volume performed within 2 weeks before beginning vaccinations and 3 weeks after the final vaccination to collect PBMCs, which were frozen for future analysis. Two patients could not have leukopheresis performed because of poor venous access. Skin tests were performed using 50 μg of the MART-1$_{27-35}$ peptide in DMSO injected intradermally in a volume of 100 μl using a tuberculin syringe and a 27-gauge needle, with 100 μl of 100% DMSO injected at a separate site as a control. Candida extract, mumps, and trichophyton provided a positive control, and saline was a negative control for assessment of DTH. At least 5 mm of induration or erythema above and beyond that shown by DMSO alone read 48 h after intradermal injection was required to score a MART-1 skin test as positive.

Dose Escalation. Patients received escalating doses of peptide with IFA, starting with the initial cohort at 300 μg/dose, then 1000 μg/dose, and 2000 μg/dose. Four patients received 300 μg, 4 received 1000 μg, and 17 patients were treated at the 2000-μg dose. The last three patients at the 2000-μg dose received 25 mg of block copolymer adjuvant CRL 1005 in addition to the IFA with the MART-1$_{27-35}$ peptide at 2000 μg.

Screening for Vitiligo and Eye Changes. All patients had a complete skin exam prior to therapy and at each visit for vaccination to screen for vitiligo. Slit lamp exams and iris photos were done by an ophthalmologist prior to starting therapy in all patients, and hand held ophthalmoscopic retinal and iris exams were performed at each vaccination visit to assess ocular toxicity. No patient had evidence of vitiligo or ocular toxicity.

Preparation of PBMC Specimens. Pheresis samples were processed to purify PBMCs by sedimentation on a Ficoll-Hypaque cushion (Pharmacia, Alameda, CA) with extensive washing in HBSS. Cells were frozen in 40% human AB serum (Gemini Bioproducts, Calabasas, CA), 50% RPMI (Life Technologies, Inc., Grand Island, NY), and 10% DMSO (Sigma) and stored in a liquid nitrogen freezer at −168°C until use.

Proliferation Assays. Assays were performed by incubating 10^5 thawed PBMCs in wells of a round-bottomed, 96-well plate (Corning, Inc., Oneonta, NY) in sextuplicate in a total volume of 200 μl of RPMI 1640 with 10% human AB serum. Various reagents were then added, and the plates were incubated in a 5% CO$_2$ incubator at 37°C for 5 days. One μCi of tritiated thymidine was then added to each well in a volume of 20 μl and again incubated at 37°C for 16 h. The contents of each well were harvested using a Skatron harvester and counted in a liquid scintillation β counter. Results are presented as the mean of five to six determinations/point.

CASTA is a preparation of proteins derived from *Candida albicans* obtained from Greer Labs (Lenoir, NC). PHA was obtained from Sigma. Peptides used for *in vitro* studies were synthesized at the USC/Norris Cancer Center Core Peptide Facility.

Cytokine Assays. Assays were performed using peptide-stimulated T cells as effector cells. Peptide-stimulated T cells were produced by incubating 2 × 10^5 thawed PBMCs with

MART-1$_{27-35}$ or FLU-MI peptide-pulsed dendritic cells that were irradiated with 6000 rads at a 1:3 ratio in wells of a 24-well plate (Corning). Cells were plated in IMEM media with 10% human AB serum. Two days later, IL-2 (kindly provided by Chiron, Emeryville, CA) was added at 50 IU/ml. Fresh IL-2 was added every 3–4 days. After 10 days, the T cells were restimulated with thawed autologous PBMCs pulsed with 10 μg/ml of MART-1$_{27-35}$ peptide at 37°C for 2 h and irradiated with 3000 rads. IL-2 was again added 48 h later at 50 IU/ml, T cells were restimulated with peptide-pulsed PBMCs every 7 days, and after four restimulations were harvested for immune assays. The performance of cytokine release assays after two or three restimulations invariably resulted in high nonspecific backgrounds. For the cytokine release assay, 10^5 peptide-stimulated T cells were harvested at least 5 days after the last restimulation and incubated with 10^5 T2 cells pulsed with 10 μg/ml MART-1 peptide or 624-mel cells as targets in a total volume of 1 ml of RPMI medium without serum for 18 h in a 5% CO_2 incubator at 37°C. Neither the effectors nor the targets were irradiated. Supernatants were collected, spun briefly at 14,000 × g to pellet cells and debris, and frozen at −80°C until assays were done. IFN-γ was detected in supernatants using an antihuman IFN-γ Quantikine ELISA kit (R and D Systems, Minneapolis, MN).

ELISPOT Assays. Assays were performed with 300,000, 100,000, 30,000, and 10,000 effectors/well, with a constant 100,000 targets in triplicates. The effectors were bulk CTLs after one or two restimulations *in vitro* with peptide-pulsed antigen-presenting cells. Nitrocellulose 96-well plates were coated with anti-IFN-γ antibodies and incubated overnight at room temperature. Plates were washed and incubated at 37°C with blocking buffer. T2 target cells (10^5) pulsed with peptides were added to the wells, and then serial dilutions of effectors were added for a total volume of 200 μl/well. The plate was incubated overnight at 37°C and then washed extensively. Biotinylated secondary anti-IFN-γ antibody was added, and the plate was incubated overnight at 4°C. Plates were again washed extensively, and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reagent followed by Streptavidin alkaline-phosphatase was added. The reaction was halted by washing under running distilled water, and the plates were dried overnight at room temperature. Spots were enumerated by counting under a microscope using a computer-controlled mechanical stage and a digital camera (Olympus Optical, Kagoshima, Japan) input to a Micron 2000 Pentium II computer using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). Counts were the means of triplicates.

Statistics. The association between post-vaccine ELISA cytokine release and time to relapse was calculated using the post-vaccine level of IFN-γ or the difference of post-vaccine minus pre-vaccine levels of IFN-γ released as continuous variables. Kaplan-Meier plots were constructed, and the log-rank test was used to calculate *P*s.

RESULTS

Demographics. A total of 25 patients with stages IIB, III, and IV resected melanoma were treated in this Phase I trial. The demographic details of this group of high-risk patients is shown in Table 1. The median age of the 12 men and 13 women was

Table 1 MART-1 vaccine trial demographics

Total number of patients	25
Male	12
Female	13
Median age (yr)	52
Median time since primary diagnosis (yr)	2.5
Stage of disease	
IIB	5
III	15
IV	5
Prior therapy	
Surgery	25
Chemotherapy	2
Biologic therapy	10
Radiation therapy	5
Anergy to skin test reagents pre-vaccine	12/25
Post-vaccine MART-1 DTH skin test	15/25[a]
Patients who developed positive MART-1 skin test who were anergic pre-vaccine	7/13
Patients with positive MART-1 ELISA assay who had positive MART-1 skin test	6/11

[a] Three of 25 patients had positive MART-1 skin tests pre-vaccine; one became negative, and the other two, who remained positive, are included in the total of 15 positive.

52. Fifteen patients had resected stage III disease, mostly lymph nodal recurrences after adjuvant IFN therapy, and 5 each had resected stage IIB or IV disease. Twenty-one had cutaneous melanoma, and 4 had ocular melanoma. The median time since diagnosis of the primary lesion for the whole group was 2.5 years. Eight of the patients had failed previous IFN-α, and two had a cellular vaccine. Three patients failed to be leukopheresed after finishing the series of four vaccinations, one because of progressive disease, and two because of inadequate venous access, leaving 22 patients with blood samples collected for evaluation both before and after vaccination.

Toxicities. All 25 patients were evaluable for toxicity. The overall toxicities of the MART-1$_{27-35}$ vaccine are shown in Table 2. The MART-1$_{27-35}$ vaccine was generally well tolerated, but almost all patients (23 of 25) had grade I or II local tenderness and pain at the injection sites. Fifteen patients developed granulomata at the injection sites, although none needed to be resected because of symptoms. One patient had a granuloma resected because of suspicion that it might represent a recurrence, and effector cells were grown from the resected granuloma tissue for a cytokine assay to determine antigen specificity as described below. Fatigue was observed in 14 patients but was not dose related. The only grade III toxicities were neutropenia that was transient and not felt to be vaccine related in one patient at the 1000-μg dose and grade III nausea in one patient at the 2000-μg dose level. Nausea was observed in five patients, and fevers, invariably low grade, were observed in three patients. Six patients experienced headaches, and six had arthralgia. Toxicity was not dose related, with no appreciable differences noted between the 300-, 1000-, and 2000-μg doses. Patients were screened for vitiligo and ocular toxicity as indicated in "Materials and Methods," and none were observed. In conclusion, the toxicity of the MART-1$_{27-35}$/IFA vaccine was modest and not dose related, and the vaccine was felt to be well tolerated, with only one episode of grade III vaccine-related toxicity. Greater and more prolonged granuloma formation ap-

Table 2 MART-1 toxicities[a]

	Level 1 (n = 4)		Level 2 (n = 10)		Level 3 (n = 8)		Level 4 (n = 3)	
	1/2[b]	3/4[b]	1/2[b]	3/4[b]	1/2[b]	3/4[b]	1/2[b]	3/4[b]
Allergy					1		1	
Fever			2		1			
Granulocytopenia				1	1			
Arthralgia	2		2		1		1	
Fatigue	4		5		4		1	
Headache			3		2		1	
Thrombocytopenia			1					
Nausea	1		1		1	1	1	
Diarrhea	2		1				1	
SGOT					2			
SGPT					2			
Granuloma	1		6		5		3	
Dermatologic			2		1		1	
Local pain	4		8		8		3	
Ocular								
Vitiligo								
Autoimmune								

[a] Level 1, 300 μg MART-1 + IFA every 3 weeks × 4 doses; Level 2, 1000 μg MART-1 + IFA every 3 weeks × 4 doses; Level 3, 2000 μg MART-1 + IFA every 3 weeks × 4 doses; and Level 4, 300 μg MART-1 + CRL 1005 every 3 weeks × 4 doses.

[b] Toxicity/grade.

Table 3 Proliferation of PBMC pre- and post-vaccine

Frozen PBMCs were thawed and added to 96-well plates as described in "Materials and Methods," and either complete medium, PHA at 1 μg/ml, MART-1$_{27-35}$ at 1 and 10 μg/ml, or CASTA at 5 μg/ml were added for 5 days. One microcurie of tritiated thymidine was added to each well for 18 h, and the wells were harvested and counted using a beta scintillation counter. Incorporation of tritiated thymidine is indicated. PMBCs from six healthy volunteers were used to establish the normal values (not shown).

	Pre-vaccine[a]	Post-vaccine[a]
Complete medium	6252 ± 1957	7140 ± 2451
PHA	84,163 ± 20,610	89,634 ± 15,901
MART-1 μg/ml	7047 ± 2367	7892 ± 2491
MART-10 μg/ml	6567 ± 2011	8188 ± 2536
ACTG-5 μg/ml	37,835 ± 13,265	45,411 ± 10,883

[a] Data are means of quintuplicate counts per minute of incorporated tritiated thymidine ± SD.

peared to occur in the three patients that received the block copolymer CRL 1005 added to the MART-1$_{27-35}$/IFA vaccine.

DTH Skin Test Results. Skin test reactivity to a panel of recall antigens was assessed prior to and after MART-1$_{27-35}$ vaccine therapy. The data at the bottom of Table 1 show that, surprisingly, 12 of 25 patients tested were anergic to recall antigens prior to MART-1$_{27-35}$ vaccination. DTH to the MART-1$_{27-35}$ peptide was also assessed by skin testing, and 2 of 25 patients reacted to the vaccine peptide prior to vaccination. Twenty-five patients were tested after MART-1$_{27-35}$ vaccination for DTH to the peptide after baseline reactivity to DMSO was subtracted as background, and 15 of 25 were positive, *i.e.*, 13 of 25 developed antigen-specific DTH reactivity after vaccination, defined as at least 5 mm of induration and/or erythema after subtraction of the DMSO alone control, including seven patients who were anergic to the panel of recall antigens (*Candida*, mumps, and trichophyton) prior to and after vaccination. Skin test reactivity to the MART-1$_{27-35}$ peptide did not appear to be dose related, nor did it correlate in any way with relapse of disease or survival (data not shown). As shown in Table 1, 6 of 11 patients with an immune response by ELISA also had a positive skin test to MART-1$_{27-35}$.

Proliferation Assays. Proliferation of patient PBMCs in response to MART-1$_{27-35}$ was tested prior to and after the series of four vaccinations to assess whether a proliferative response had been successfully induced to that class I-restricted peptide. Immune parameters were measured in the 25 patients that received the MART-1$_{27-35}$/IFA vaccine. The proliferation of PBMCs in response to PHA, a mitogenic stimulus, as well as in response to CASTA, a *C. albicans* protein extract, was also assessed as an overall measure of immune status prior to and after vaccination. The results are shown in Table 3, in which 24 patients were tested, showing for the whole group no overall

evidence of a proliferative response to the MART-1$_{27-35}$ peptide when pulsed onto PBMCs at 1 μg/ml (7047 ± 2367 to 7892 ± 2491 cpm) or 10 μg/ml (6567 ± 2011 to 8188 ± 2536 cpm) without further restimulation. No changes were seen in proliferative responses to PHA (84,163 ± 20,610 to 89,634 ± 15,901 cpm) or *C. albicans* proteins (37,835 ± 13,265 to 45,441 ± 10,883 cpm). In several cases, the proliferation of PBMCs to MART-1$_{27-35}$ peptide decreased appreciably after vaccination, without a clear reason.

Cytokine Release Immune Assays in MART-1-vaccinated Patients. MART-1$_{27-35}$-specific immunity was measured in 22 patients who had pre- and post-vaccination PBMC samples available by measuring antigen-specific release of IFN-γ by ELISA from effector cells restimulated weekly four times with peptide-pulsed irradiated PBMC stimulators. Effectors were incubated for 18 h with control HLA-A2+ T2 cells, MART-1$_{27-35}$ peptide-pulsed T2 cells, or 624-mel, a HLA-A2-positive, MART-1-positive melanoma cell line, as described in detail in "Materials and Methods." The results of pre- and post-vaccine cytokine release assays for those 22 patients are shown in Table 4. A total of 11 of 22 patients, at all dose levels, showed evidence of increased reactivity to MART-1$_{27-35}$ peptide-pulsed T2 targets or MART-1-positive target 624-mel after vaccination, with release of IFN-γ secreted per 10^5 cells/ml that ranged from 100 to 3000 pg/ml. The cytokine release had to be at least 100 pg/ml above the T2 unpulsed control to be scored as positive, which represented two SDs from the mean of the T2 unpulsed controls. Two patients at the 300-μg dose level, 1 at the 1000-μg dose level, and 6 at the 2000-μg dose level, including one of three who had the block copolymer added to their vaccine, had increased ELISA reactivity to MART-1$_{27-35}$-pulsed T2 cells. Two additional patients, one each at the 300- and 1000-μg cohorts, had reactivity to 624-mel. Seven of nine ELISA responders had increased cytokine release to both T2 cells pulsed with the MART-1$_{27-35}$ peptide and 624-mel cells. Four patients, including two responders by ELISA, had detectable reactivity prior to vaccination. All of the cytokine release assays were repeated at least once, with similar results. For cytokine release assays, background release of unpulsed targets incubated with effectors ranged from 0 to 60% of MART-1 peptide-pulsed targets incubated with effectors and are shown in

Table 4 Immune response to MART-1 vaccination: Release of IFN-γ by peptide-stimulated effector cells pre- and post-vaccination

Effector cells were prepared as described in "Materials and Methods" by restimulation of PBMCs in the presence of MART-1_{27-35} peptide-pulsed antigen-presenting cells. 100,000 resulting effector cells were plated after the fourth restimulation *in vitro* with 100,000 targets consisting of either HLA-A2+ T2 cells or T2 cells pulsed with MART-1_{27-35} peptide (peptide at a concentration of 10 μg/ml) in a 24 well plate in complete medium for 18 h in a volume of 1 ml. The supernatant was harvested and then spun in a microcentrifuge at 14,000 × *g* for 30 s to pellet cells and debris. Supernatants were removed and used to measure IFN-γ release using a commercial ELISA kit as described in "Materials and Methods." Figures shown are the means of duplicate values of IFN-γ. Similar results were obtained in repeated experiments for each patient.

Dose	Level	Patient no.	T2 unpulsed targets[a]	T2 MART-1 pulsed targets[a]	624-mel[a]
300	Pre	1	390	1650	1260
	Post		0	3300[b]	2025[b]
300	Pre	2	160	0	35
	Post		165	35	250[b]
300	Pre	3	0	0	0
	Post		0	0	0
1000	Pre	4	168	342	210
	Post		180	2880[b]	1800[b]
1000	Pre	5	96	16	96
	Post		215	23	670
1000	Pre	6	0	117	170
	Post		0	912[b]	468[b]
2000	Pre	7	80	0	245
	Post		10	118[b]	160
2000	Pre	8	10	10	0
	Post		0	0	0
2000	Pre	9	0	0	0
	Post		0	90	0
2000	Pre	10	21	40	0
	Post		0	416[b]	299[b]
2000	Pre	11	15	10	0
	Post		0	10	0
2000	Pre	12	212	88	251
	Post		233	1583[b]	908[b]
2000	Pre	13	80	46	0
	Post		53	24	50
2000	Pre	14	0	2350	779
	Post		205	1065	616
2000	Pre	15	0	19	0
	Post		0	0	0
2000	Pre	16	0	0	1060
	Post		0	0	865
2000	Pre	17	0	0	0
	Post		0	2580[b]	273[b]
2000	Pre	18	52	21	336
	Post		22	0	40
2000	Pre	19	0	0	43
	Post		442	688[b]	737[b]
2000	Pre	20[c]	0	0	0
	Post		0	10	50
2000	Pre	21[c]	350	300	250
	Post		450	90	230
2000	Pre	22[c]	96	24	62
	Post		50	163[b]	0

[a] Data shown are pg of IFN-γ secreted per 10^5 cells in 24 h.
[b] Vaccine responses.
[c] Patients who received CRL 1005 block copolymer.

the leftward column of Table 4; the middle column represents actual MART-1-specific release without subtraction of any background. The observation that reactivity was seen with

MART-1_{27-35} peptide-pulsed T2 targets as well as HLA-A2-positive, MART-1-positive cell line 624-mel suggested that the increased immune effectors detected in the peripheral blood could recognize naturally processed MART-1_{27-35} peptide on the surface of a tumor cell line. To verify that increased MART-1_{27-35}-specific reactivity measured by specific cytokine release after vaccination was antigen specific, and that patients showing no or minimal (<100 pg/ml) cytokine release could react to an influenza stimulus, PBMCs from selected patients who had evidence either of a clearly positive response or no change in MART-1_{27-35}-specific cytokine release were subjected to a cross-specificity assay in which pre- and post-vaccine PBMC samples were split into two and stimulated as above four times weekly in the presence of a HLA-A2-restricted influenza virus matrix protein or MART-1_{27-35}-peptide pulsed onto irradiated PBMC stimulators and then used as effectors in a cytokine release assay as in Table 4, with targets consisting of T2 cells pulsed with FLU peptide or T2 cells pulsed with the MART-1_{27-35} peptide. A positive signal in FLU-specific release and no MART-1 specific signal was expected for FLU-stimulated effector cells both pre- and post-vaccination for all patients. No FLU-specific reactivity was expected for MART-1_{27-35}-stimulated effector cells for any patients pre- or post-vaccination, but increased MART-1_{27-35}-specific reactivity by MART-1_{27-35}-stimulated effector cells after vaccination was expected for patients who had an immune response in Table 4. In the *left panel* of Fig. 1, for the pre- and post-vaccine sample pair from patient 6, FLU-specific cytokine release was observed both pre- and post-vaccine, but significant MART-1_{27-35}-specific reactivity was seen only after vaccination, as shown also in Table 4. The data in the *right panel* of Fig. 1 suggest that for the pre- and post-vaccine sample pair from patient 11, there was no MART-1_{27-35}-specific response post vaccine, reproducing the result in Table 4. but that good FLU-specific release was observed pre- and post-vaccine as a positive control. These data confirm the MART-1 specificity of the cytokine release data shown in Table 4 for patients 6 and 11 in a repeated experiment and demonstrate that patients without a MART-1_{27-35} response still have the ability to mount a FLU-specific immune response.

ELISPOT Immune Assays in MART-1-vaccinated Patients. As a further measure of immune response to the MART-1_{27-35} peptide vaccine, we devised an ELISPOT assay that detected the presence of single E:T cell interactions by immobilization of E:T pairs and detection of IFN-γ after two restimulations with peptide-pulsed stimulators *ex vivo*. This assay did not directly measure antigen-specific IFN-γ-releasing effector cells in fresh blood but yielded a semiquantitative assessment of the presence of antigen-specific effector cells after minimal restimulation. The data in Table 5 show that 11 of 20 patients tested demonstrated an immune response to MART-1_{27-35} after vaccination, although background reactivities to FLU peptide-pulsed or unpulsed T2 targets were higher than in the cytokine release assay, and not all patients that had evidence of boosted immunity by ELISA also had a positive response by ELISPOT. These data do support the notion that an augmented MART-1_{27-35}-specific CTL response can be detected in peripheral blood cells after vaccination with the MART-1_{27-35} peptide plus adjuvant with a quantitative single-cell ELISPOT assay. However, only 3 of 11 ELISPOT responders had a response by
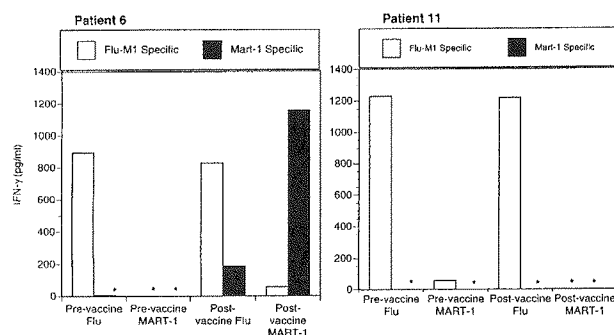
Fig. 1 The experiment in Fig. 1 describes a cross specificity analysis of PBMC samples from one patient with a positive immune response (*Patient 6*) and one patient with a negative response (*Patient 11*) from Table 4. Effector cells were prepared as described in "Materials and Methods" by restimulation of PBMC samples that were split into two aliquots. One aliquot was restimulated in the presence of HLA-A2+ FLU peptide-pulsed stimulator cells, and the other was restimulated in the presence of MART-1$_{27-35}$ peptide-pulsed stimulator cells. One hundred thousand resulting effector cells were plated after the fourth restimulation *in vitro* with 100,000 targets consisting of either HLA-A2+ T2 cells pulsed with the FLU peptide or T2 cells pulsed with MART-1$_{27-35}$ peptide (both peptides at a concentration of 10 μg/ml overnight at 37°C) in a 24-well plate in complete medium for 18 h in a volume of 1 ml. The supernatant was harvested and then spun in a microcentrifuge at 14,000 × g for 30 s to pellet cells and debris. Supernatants were removed and used to measure IFN-γ release using a commercial ELISA kit as described in "Materials and Methods." Figures shown are the means of duplicate values of IFN-γ. Similar results were obtained in repeated experiments for both patients. *Left, Patient 6* (a responder to the MART vaccine): the *first pair* of *columns* from the *left* shows the cytokine release response to FLU restimulated PBMCs pre-vaccination (*Pre-vaccine Flu*) by either FLU-pulsed T2 targets (☐) or MART-1$_{27-35}$-pulsed targets (■). *, zero level of cytokine release. The cytokine release response to MART-1$_{27-35}$-restimulated PBMCs for the two targets is shown in the second group (*Pre-vaccine MART-1*), and for FLU or MART-1$_{27-35}$-restimulated PBMCs post-vaccine (*Post-vaccine Flu* or *Post-vaccine MART-1*) in which a FLU-specific response was detected pre- and post-vaccine, as shown, and where a MART-1-specific response was detected post- but not pre-vaccine Similar data are shown for MART-1 nonresponding patient 11 (*right*), in which a FLU-specific response was detected pre- and post-vaccine, as shown, but no response to MART-1$_{27-35}$.

ELISA, and 6 of the 12 ELISPOT responders have relapsed, with a median of 16 months of follow-up. There was no relationship between ELISPOT response and time to relapse.

Correlation between Immune Response and Relapse-free Survival. Although it was not a prospectively determined end point of this Phase I study, a correlation was made between relapse-free survival and immune response by cytokine release ELISA at the median of 16 months of follow-up, at which time 3 of 25 patients had died and 16 of 25 remained free of disease. The association between the two continuous variables was calculated using the log-rank test, indicating that relapse-free survival time correlated with post-vaccine ELISA assay ($P < 0.003$) and also was associated with the difference between pre- and post-vaccine values ($P < 0.04$). When the patients were arbitrarily grouped into those with a "strong" cytokine response (>100 pg/ml post-vaccine compared with pre-vaccine), "weak" cytokine response (<100 pg/ml post-vaccine compared with pre-vaccine), or no re-

sponse (0 pg/ml post-vaccine compared with pre-vaccine), it is intriguing to note that all eight patients in the "strong" group were alive and free of progression, whereas the nine relapsed patients (including the three who had died from disease) were distributed between the no response or "weak response" groups.

DISCUSSION

Endogenously synthesized antigens of virtually all mammalian cells are processed and converted to small epitope peptides that are displayed on the cell surface in association with class I MHC molecules (21–24). Peptides bind with different consensus motifs based on preferred NH$_2$ (positions 1 and 2) and COOH (positions 8, 9, and 10) amino acids that localize the peptide to the MHC "cleft" or peptide-binding groove (25–27). The binding ability of epitope peptides define class I MHC restriction and T-cell receptor specificity for any protein. Tumor-associated or tumor-specific antigenic proteins present MHC-restricted peptide epitopes at the cell surface for T-cell recognition (28). To provide a strong antigen-specific stimulus in this vaccine trial, we used an epitope peptide derived from MART-1, an melanoma antigen recognized by T cells (10, 11). The principal goal of this immunization strategy was to augment antigen-specific T-cell responses in patients to eliminate tumor and prevent relapse in individuals with microscopic tumor burdens, as has been shown in experimental murine models.

Antigens present on melanomas can be broadly divided into three categories; one is the cancer/testis group expressed by a large variety of tumors, of which the *MAGE, BAGE,* and *GAGE* gene families are examples (29). The second is a group of mutated normal genes uniquely present on individual tumors; β-catenin, HLA-A variants, and p16 are examples (19, 20). The third category is differentiation antigens that are expressed by melanomas as well as normal melanocytes; MART-1/Melan A, tyrosinase, gp75, TRP-2, and gp100/pMel 17 are examples (10–17). The tumor-restricted distribution of the first two groups make them attractive targets for immunotherapy, but there is little evidence of immune reactivity to those antigens in most melanoma patients. In contrast, the differentiation antigens, although expressed in normal tissue, clearly provoke an immune response in melanoma patients. Cytolytic T cells from peripheral blood, or which infiltrate tumors from HLA-A2-positive patients, recognize an antigen or group of antigens on HLA-A2 melanoma cells and fresh tumors (6, 30–32). MART-1 was defined as a gene product recognized by CTL clones from peripheral blood of a melanoma patient and by CTLs derived from a melanoma patient's TILs, in whom cellular therapy had induced a partial regression of metastatic disease (10, 11), suggesting that it might be a target recognized by T cells with antitumor potential. The TILs that recognized MART-1 as well as TILs from a number of other melanoma patients reacted with virtually all melanoma cell lines that expressed HLA-A2, and transfection of the *A2* gene into other non-A2-expressing melanoma lines increased their sensitivity to TIL lysis (33). This suggested that MART-1 was a common A2-restricted melanoma antigen recognized by CTLs. MART-1 was expressed by virtually all metastatic melanoma lesions, a majority of cells lines

Table 5 Immune response by ELISPOT to MART-1 vaccination

Effector cells were prepared as described in "Materials and Methods" by restimulation of PBMCs in the presence of MART-1$_{27-35}$ peptide-pulsed stimulator cells. After the second restimulation *in vitro*, 10,000 or 30,000 resulting effector cells were plated with 10,000 HLA-A2+ T2 cells, T2 cells pulsed with a HLA-A2 restricted FLU peptide, or T2 cells pulsed with the HLA-A2 restricted MART-1$_{27-35}$ peptide on nitrocellulose filters as described in "Materials and Methods," and an ELISPOT assay was performed. The mean number of spots enumerated by a computerized digital imaging system that automatically counted spots per well in triplicate for each number of effectors per 10,000 targets is shown for paired samples pre- and postvaccine.

Dose	Level	Patient no.	No peptide[a]		MART-1[a]		FLU[a]	
			3 × 10e4	10e4	3 × 10e4	10e4	3 × 10e4	10e4
300	Pre	2	186	110	282	174	222	160
	Post		230	145	220	155	223	133
300	Pre	3	57	40	171	84	40	14
	Post		122	53	303[b]	147[b]	76	52
1000	Pre	5	90	60	111	103	138	89
	Post		188	199	223[b]	157[b]	101	85
1000	Pre	6	200	119	105	55	291	94
	Post		210	172	85	76	446	211
2000	Pre	7	94	47	268	92	219	78
	Post		160	62	69	40	64	17
2000	Pre	8	81	29	217	52	266	42
	Post		137	8	28	9	12	13
2000	Pre	9	69	42	197	67	ND[c]	ND
	Post		164	81	156	82	ND	ND
2000	Pre	10	79	65	78	114	221	138
	Post		160	96	>500[b]	454[b]	105	77
2000	Pre	11	82	88	88	86	84	57
	Post		258	58	223[b]	157[b]	101	85
2000	Pre	12	52	17	225	145	18	11
	Post		40	7	287[b]	298[b]	50	23
2000	Pre	13	98	41	230	92	127	157
	Post		132	41	318[b]	202[b]	41	25
2000	Pre	14	106	56	346	184	173	97
	Post		207	117	391[b]	244[b]	129	64
2000	Pre	15	95	61	253	149	227	106
	Post		222	108	246[b]	322[b]	86	41
2000	Pre	16	5	7	128	4	50	13
	Post		54	10	296[b]	118[b]	11	4
2000	Pre	17	103	66	248	192	152	103
	Post		167	105	329	222	124	108
2000	Pre	18	153	117	136	80	170	186
	Post		191	179	306[b]	190[b]	249	190
2000	Pre	19	34	8	85	34	43	20
	Post		91	13	63	27	17	9
2000	Pre	20[d]	59	74	122	56	137	58
	Post		103	71	122	68	94	85
2000	Pre	21[d]	64	94	141	82	111	72
	Post		112	90	224[b]	149[b]	123	70
2000	Pre	22[d]	146	97	197	96	307	96
	Post		214	75	188	99	146	83

[a] Data shown are number of spots counted by a digital imager per number of effector cells indicated at top.
[b] Vaccine response.
[c] ND, not done.
[d] Patients who received CRL 1005 block copolymer.

derived from metastatic melanomas, and also by melanocytes, but not by any other normal tissue. The *MART-1* gene encoded a putative protein of M_r 26,000 with sequences that matched the known HLA-A2 binding motifs. The nonamer sequence AA-GIGILTV, representing residues 27–35 of the MART-1 protein, bound most strongly to HLA-A2 (34). This peptide stimulated the growth of specific CTLs from the PBMCs of melanoma patients and of normal persons (35). Multiple restimulations of PBMCs with MART-1$_{27-35}$ peptide resulted in cultures of MART-1-specific CTLs derived from 11 of 12 melanoma patients (36). These CTLs lysed fresh uncultured melanoma cells

and were 100-fold more lytically active against melanoma cells than TILs grown in high-dose IL-2. The majority of TILs grown from patients with melanoma are capable of recognizing the MART-1$_{27-35}$ peptide, and some of those TIL cultures induced regression of metastatic melanoma after adoptive transfer with IL-2. The repertoire of Vβ T-cell receptor molecules from TILs and peripheral blood-derived CTL lines that are MART-1 specific are quite skewed (37–39).

Peptides derived from MART-1 were eluted from melanoma cells, suggesting that MART-1$_{27-35}$ is a naturally occurring antigen on fresh tumors (40–42). A protein database anal-

ysis demonstrated that sequences conforming to the MART-1 A2 binding motif and possessing features important for CTL recognition occurred frequently in proteins (43), and that a peptide derived from glycoprotein C of herpes simplex virus could sensitize target cells to lysis by MART-1$_{27-35}$-specific CTLs (43). These data suggest that epitope mimicry by normal or other commonly occurring proteins may account for the frequency of CTLs detected against melanoma antigens like MART-1.

Greater MART-1$_{27-35}$-reactive CTL activity has been demonstrated in the peripheral blood of melanoma patients compared with normal persons, suggesting that a tumor-related "priming" effect has occurred (44), and in a clinical trial of MART-1$_{27-35}$ peptide with adjuvant in patients with metastatic melanoma, a boost in MART-1$_{27-35}$-specific immunity was observed in a significant proportion of patients, but without clinical responses (45). Clinical benefit for a MART-1 peptide vaccine has been observed in a trial that included multiple peptides with granulocyte/macrophage-colony stimulating factor for metastatic melanoma, with 5 of 26 patients showing a clinical response that correlated with augmented MART-1-specific CTLs in at least three cases (46). The MART-1 peptide was used with several other peptides to pulse autologous dendritic cells, which were adoptively transferred by intralymph nodal and s.c. injections, resulting in a 25% response rate in patients with metastatic melanoma (47).

The overlapping MART-1$_{26-35}$ peptide has been shown to be more immunogenic than the 27–35 epitope, and a single amino acid modification to the 26–35 peptide rendered it a stronger binder to A2.1 and even more immunogenic (48, 49). This peptide is a prime candidate for future clinical vaccine trials. The A2.1-restricted peptide in this study has been shown to bind to multiple other HLA-A2 subtypes, as well as allele *A45*, but no other MART-1-specific peptides have been shown to elicit specific immune responses *in vitro* in patients bearing other HLA class I alleles (50–53).

Western blotting as well as immunohistochemical staining using MART-1 antibodies have established that MART-1 is a transmembrane protein component of the melanosome complex (54, 55). Reverse transcription-PCR analysis has shown that MART-1 mRNA is present in virtually 100% of metastatic melanoma lesions, yet immunohistochemical staining has shown that there is considerable heterogeneity in MART-1 expression on primary and metastatic lesions, with 60–90% of all lesions staining positively (56–60). In one study, deletion of MART-1 expression, as well as transporter associated with antigen processing (TAP) transporter expression, rendered cells transparent to CTL recognition, suggesting that loss of MART-1 may be a mechanism for immune evasion (61).

The data presented in this report suggest that ~50% of patients have demonstrated augmented, antigen-specific T-cell reactivity after receiving a MART-1$_{27-35}$/IFA vaccine. The use of cytokine release assays with IFN-γ and the use of an automated ELISPOT assay yielded semiquantitative information about increases in antigen-specific effector cells in circulating PBMCs after vaccination. The cross-specificity ELISA data from selected patients suggest that the CTLs generated from post-vaccine PBMCs are truly antigen specific, but the ELISPOT data are consistent with a fairly low frequency of precursor

CTLs after vaccination. A statistical analysis of the IFN-γ ELISA data, albeit with small numbers, provided a provocative hint that there was a correlation between ELISA response and relapse-free survival. The correlation of antigen-specific ELISA assay with a desired clinical end point is encouraging. However, there was no clear relationship between DTH reactivity or ELISPOT response and relapse-free survival, which suggests a cautious interpretation for the data. No increases in MART-1$_{27-35}$-specific proliferation were seen after one restimulation of PBMCs, also consistent with a low frequency of antigen-specific T cells.

The MART-1 antigen is also expressed by normal melanocytes (31, 62), and the use of a MART-1$_{27-35}$ epitope peptide vaccine had the potential to induce autoimmune reactions. It is not known whether normal melanocytes effectively present the MART-1$_{27-35}$ epitope peptide to T cells *in vivo*, and previous clinical experience with the adoptive transfer of CTLs that were highly MART-1 reactive and mediated regression of tumor did not indicate the onset of any autoimmune damage to skin, brain, inner ear, or retina, where melanocyte lineage cells are located. As in the present study, patients with metastatic melanoma that received a MART-1$_{27-35}$ peptide vaccine did not demonstrate any evidence of ocular or other toxicity.[4] None of the 25 patients with resected stages IIB/III/IV melanoma that received MART-1$_{27-35}$ peptide vaccine with Montanide ISA-51 in the present study exhibited vitiligo, which has been observed in melanoma patients receiving immunotherapy with IL-2 or chemotherapy combined with IL-2 (63). No ocular problems nor any evidence of autoimmune pathology have occurred in any patients on this trial with a median follow-up of 16 months. Toxicity was confined to mostly local pain, edema, and formation of granuloma, none of which became infected or required surgical intervention.

None of the three patients who died and none of nine relapsed patients showed evidence of increased immunity to the MART-1$_{27-35}$ vaccine. Eleven of 16 patients who are free of disease showed an immune response, as evidenced by increased release of IFN-γ after exposure of PBMCs to MART-1$_{27-35}$ peptide-pulsed antigen presenting cells or MART-1-expressing tumor cell line 624-mel. To determine whether augmented MART-1$_{27-35}$-specific release of IFN-γ post-vaccination was associated with prolonged time to relapse, we used Kaplan-Meier plots and the log-rank test. The log-rank test based on this model was used with cytokine values grouped into thirds prior to the analysis to calculate P for the significance of the association. P between the level of immune response post-vaccine and relapse-free survival was 0.01, and for the difference between pre- and post-vaccine cytokine values, P was 0.009. The number of vaccinated patients is too small to draw a statistically meaningful general conclusion from the lack of immune responses in relapsed patients. The data in this trial, however, support the idea of a follow-up trial using multiple peptides derived from MART-1, gp100, and tyrosinase, which will be used to vaccinate high-risk melanoma patients rendered free of disease with a more prolonged schedule of immunizations in association with novel adjuvants (64, 65). An important clinical end point of a

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[4] F. Marincola, personal communication.

larger follow-up trial will be the correlation between immune response and time to relapse to determine whether augmented peptide-induced immunity has the potential to result in clinical benefit.

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